Amplite™ Fluorimetric NADH Assay Kit

Red Fluorescence

Ordering Information:	*Storage Conditions:*	*Instrument Platform:*
Product Number: 15261 (400 assays)	Keep in freezer Avoid exposure to light	Fluorescence microplate readers

Introduction

Nicotinamide adenine dinucleotide (NAD+) and nicotinamide adenine dinucleotide phosphate (NADP+) are two important cofactors found in cells. NADH is the reduced form of NAD+, the oxidized form of NADH. NAD forms NADP with the addition of a phosphate group to the 2' position of the adenyl nucleotide through an ester linkage. The traditional NAD/NADH and NADP/NADPH assays are done by monitoring the changes in NADH or NADPH absorption at 340 nm. This method suffers low sensitivity and high interference since the assay is done in the UV range that requires expensive quartz microplates.

This Amplite™ NADH Assay Kit provides a convenient method for the detection of NADH. The enzymes in the system specifically recognize NADH in an enzyme recycling reaction. In addition, this assay has very low background since it is run in the red visible range that significantly reduces the interference resulted from biological samples. The assay has demonstrated high sensitivity and low interference at Ex/Em = 540/590 nm.

NADH + Amplite™ NADH Sensor (Non-fluorescent) ⟶ **NAD + Red Fluorescent Product**

The Amplite™ Fluorimetric NADH Assay Kit provides a sensitive, one-step fluorimetric assay to detect as little as 3 nanomoles of NADH in a 100 µL assay volume (0.3 µM; Figure 1). The assay can be performed in a convenient 96-well or 384-well microtiter-plate format and easily adapted to automation without a separation step. Its signal can be easily read by either a fluorescence microplate reader at Ex/Em = 530 to 570/590 to 600 nm (maximum Ex/Em = 540/590 nm) or an absorbance microplate reader at 576±5 nm.

Kit Key Features

Broad Application:	Can be used for quantifying NADH in solutions and in cell extracts.
Sensitive:	Detect as low as 10 nanomoles of NADH in solution.
Continuous:	Easily adapted to automation without a separation step.
Convenient:	Formulated to have minimal hands-on time. No wash is required.
Non-Radioactive:	No special requirements for waste treatment.

Kit Components

Components	Amount
Component A: NADH Recycling Enzyme Mixture	2 bottles (lyophilized powder)
Component B: NADH Assay Buffer	1 bottle (20 mL)
Component C: NADH Standard	1 vial (142 µg)

Assay Protocol for One 96-Well Plate

Brief Summary

Prepare NADH reaction mixture (50 µL) → Add NADH standards or test samples (50 µL) → Incubate at room temperature for 15 min-2hr → Read fluorescence intensity at Ex/Em = 540/590 nm

Note: Thaw one of each kit component at room temperature before starting the experiment.

Ordering: sales@aatbio.com; 800-990-8053 or 408-733-1055; Fax: 408-733-1304
Technical Support: support@aatbio.com; 408-733-1055

1. Prepare NADH stock solution:

Add 200 µL of PBS buffer into the NADH standard vial (Component C) to make 1 mM (1 nmol/µL) stock solution.

Note: The unused NADH solution should be divided into single use aliquots and stored at -20°C.

2. Prepare NADH reaction mixture:

Add 10 mL of Amplite™ NADH Assay Buffer (Component B) to the bottle of NADH Recycling Enzyme Mixture (Component A), and mix well.

Note: This solution is enough for two 96-well or four 384-well plates. The unused NADH reaction mixture should be divided into single use aliquots and stored at -20°C.

3. Prepare serial NADH (0 to 100 µM) solutions:

3.1 Add 50 µL of NADH standard stock solution (from Step 1) to 450 µL PBS (pH 7.4) buffer to generate 100 µM (100 pmol/µL) standard.

Note: Diluted NADH standard solution is unstable, and should be used within 4 hours.

3.2 Take 200 µL of 100 µM solution to perform 1:3 serial dilutions to get 30, 10, 3, 1, 0.3, 0.1 and 0 standard NADH solutions.

3.3 Add NADH standards and NADH containing test samples into a 96-well solid black microplate as described in Tables 1 and 2

Note: Prepare the cell or tissue samples as desired.

Table 1. Layout of NADH standards and test samples in a solid black 96-well microplate

BL	BL	TS	TS	….	….						
NS1	NS1	….	….	….	….						
NS2	NS2										
NS3	NS3										
NS4	NS4										
NS5	NS5										
NS6	NS6										
NS7	NS7										

Note: NS= NADH Standards, BL=Blank Control, TS=Test Samples.

Table 2. Reagent composition for each well

NADH Standard	**Blank Control**	**Test Sample**
Serial dilutions* (50 µL)	PBS: 50 µL	50 µL

**Note: Add the serially diluted NADH standards from 0.1 µM to100 µM into wells from NS1 to NS7 in duplicate.*

4. Run NADH assay in supernatants reaction:

4.1 Add 50 µL of NADH reaction mixture (from Step 2) to each well of the NADH standard, blank control, and test samples (see Step 3.3) to make the total NADH assay volume of 100 µL/well

Note: For a 384-well plate, add 25 µL of sample and 25 µL of NADH reaction mixture into each well.

4.2 Incubate the reaction at room temperature for 15 minutes to 2 hours, protected from light.

4.3 Monitor the fluorescence increase with a fluorescence plate reader at Ex/Em = 530 to 570/590 to 600 nm (optimal at Ex/Em = 540/590 nm).

Note: The contents of the plate can also be transferred to a white clear bottom plate and read by an absorbance microplate reader at the wavelength of 576±5 nm. The absorption detection has lower sensitivity compared to fluorescence reading.

Data Analysis

The fluorescence in blank wells (with the PBS buffer only) is used as a control, and is subtracted from the values for those wells with the NADH reactions. A NADH standard curve is shown in Figure 1.
Note: The fluorescence background increases with time, thus it is important to subtract the fluorescence intensity value of the blank wells for each data point.

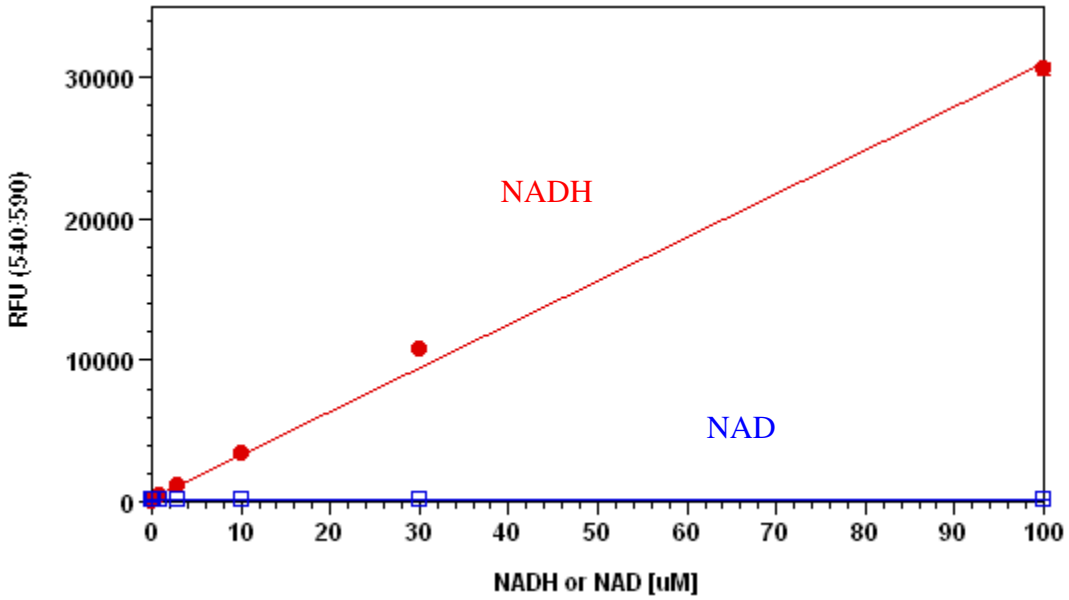

Figure 1. NADH dose response was measured with Amplite™ NADH Assay Kit in a 96-well black plate using a NOVOStar microplate reader (BMG Labtech). As low as 1 µM (10 nmol/well) of NADH can be detected with 1 hour incubation time (n=3) while there is no response from NAD.

References

1. Ziegenhorn J, Senn M, Bucher T. (1976) Molar absorptivities of beta-NADH and beta-NADPH. Clin Chem, 22, 151.
2. Ikegami T, Kameyama E, Yamamoto SY, Minami Y, Yubisui T. (2007) Structure and Properties of the Recombinant NADH-Cytochrome b(5) Reductase of Physarum polycephalum. Biosci Biotechnol Biochem.
3. Kimura N, Fukuwatari T, Sasaki R, Shibata K. (2006) Comparison of metabolic fates of nicotinamide, NAD+ and NADH administered orally and intraperitoneally; characterization of oral NADH. J Nutr Sci Vitaminol (Tokyo), 52, 142.
4. O'Donnell JM, Kudej RK, LaNoue KF, Vatner SF, Lewandowski ED. (2004) Limited transfer of cytosolic NADH into mitochondria at high cardiac workload. Am J Physiol Heart Circ Physiol, 286, H2237.